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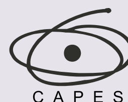

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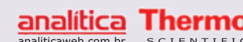

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The role of Ca^{2+}/calmodulin-dependent protein kinase II and calcineurin in TNF-α-induced myocardial hypertrophy

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Abstract

We investigated whether Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and calcineurin (CaN) are involved in myocardial hypertrophy induced by tumor necrosis factor α (TNF-α). The cardiomyocytes of neonatal Wistar rats (1-2 days old) were cultured and stimulated by TNF-α (100 µg/L), and Ca^{2+} signal transduction was blocked by several antagonists, including BAPTA (4 µM), KN-93 (0.2 µM) and cyclosporin A (CsA, 0.2 µM). Protein content, protein synthesis, cardiomyocyte volumes, $[Ca^{2+}]_i$ transients, CaMKIIδ_B and CaN were evaluated by the Lowry method, [^{3}H]-leucine incorporation, a computerized image analysis system, a Till imaging system, and Western blot analysis, respectively. TNF-α induced a significant increase in protein content in a dose-dependent manner from 10 µg/L (53.56 µg protein/well) to 100 µg/L (72.18 µg protein/well), and in a time-dependent manner from 12 h (37.42 µg protein/well) to 72 h (42.81 µg protein/well). TNF-α (100 µg/L) significantly increased the amplitude of spontaneous $[Ca^{2+}]_i$ transients, the total protein content, cell size, and [^{3}H]-leucine incorporation in cultured cardiomyocytes, which was abolished by 4 µM BAPTA, an intracellular Ca^{2+} chelator. The increases in protein content, cell size and [^{3}H]-leucine incorporation were abolished by 0.2 µM KN-93 or 0.2 µM CsA. TNF-α increased the expression of CaMKIIδ_B by 35.21% and that of CaN by 22.22% compared to control. These effects were abolished by 4 µM BAPTA, which itself had no effect. These results suggest that TNF-α induces increases in $[Ca^{2+}]_i$, CaMKIIδ_B and CaN and promotes cardiac hypertrophy. Therefore, we hypothesize that the Ca^{2+}/CaMKII- and CaN-dependent signaling pathways are involved in myocardial hypertrophy induced by TNF-α.

Key words: Myocardial hypertrophy; Tumor necrosis factor α; Calcium; Calmodulin-dependent kinase; Calcineurin

Introduction

Cardiac hypertrophy is one of the major responses of cardiomyocytes to mechanical and neurohormonal stimuli. This process results in increases in the work output of myocytes, as well as cardiac pump function. Cardiac hypertrophy is a leading cause of mortality and often progresses from the initial adaptive response of the myocardium to heart failure and ventricular dilatation (1). It is therefore essential to elucidate the molecular mechanisms underlying the development of cardiac hypertrophy.

Several studies have indicated that tumor necrosis factor alpha (TNF-α) is one of the most important factors in the induction of hypertrophy (2-5). Moreover, the direct effect of TNF-α on cardiac hypertrophy has been demonstrated in cultured cardiomyocytes. However, the molecular mechanism of TNF-α-induced cardiac hypertrophy has not yet been fully elucidated.

A number of hypertrophic stimuli increase intracellular Ca^{2+} levels (6), and reports suggest that Ca^{2+} is involved in the generation of cardiac hypertrophy (7). To date, only a few studies have been conducted investigating the effect of TNF-α on intracellular free calcium concentration and L-type calcium channels of cardiomyocytes. How TNF-α affects calcium ion movement is still an open question.

Previous studies have demonstrated that two calcium effectors, specifically Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and calcineurin (CaN), play a critical role in myocardial hypertrophy (6,8). It is uncertain whether these molecules are also involved in the cardiac hypertrophy induced by TNF-α.

In the present study, we evaluated the effects of TNF-α

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on intracellular free calcium concentration and whether the Ca^{2+}-dependent signaling, including CaMKII and CaN pathways, is involved in TNF-α-induced hypertrophy in cultured neonatal rat cardiomyocytes.

Material and Methods

Animals

The experimental protocol was approved by the Committee for the Use of Experimental Animals for Research and Teaching of China Medical University, China.

Drugs and reagents

2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-93) was used as a CaMKII inhibitor. Cyclosporin A (CsA) was used as a CaN inhibitor and BAPTA was used as an intracellular Ca^{2+} chelator. The concentrations of TNF-α (2,3,9), KN-93 (10), CsA (11), and BAPTA (6) used were selected based on previous studies. Recombinant human TNF-α was purchased from R&D (USA). KN-93, CsA, Fura-2-acetoxymethyl ester (Fura-2/AM), BAPTA, fetal calf serum, bromodeoxyuridine, transferrin, insulin, Trypsin, and Dulbecco's modified Eagle's medium (DMEM) were obtained from Sigma Chemical Co. (USA).

Culture of neonatal rat ventricular myocytes

Primary cultures of cardiomyocytes were prepared from the ventricles of neonatal Wistar rats as described by Simpson (12). Cardiomyocytes were collected for culture from 1- to 2-day-old neonatal rats and maintained at 37°C in humidified air containing 5% CO_2. After dissociation of the heart tissue with Trypsin, cells were pre-plated for 1 h onto 100-mm culture dishes in DMEM with 10% fetal calf serum to reduce the number of non-myocyte cells. Cells that were not attached to the pre-plated dishes were plated onto 6-well culture plates at a density of 1 x 10^6/mL. Non-myocytes in the cultures were limited to ≤10% of the total cell number by inclusion of bromodeoxyuridine (0.1 mM) in the medium for the first 2 days. After 24 h the culture medium was replaced with serum-free medium consisting of DMEM, transferrin (5 mg/mL), insulin (1 mg/mL), and BrdU (0.1 mM). Antagonists, including BAPTA, KN-93 and CsA, were administered 30 min before TNF-α administration, and the cells were subsequently cultured for an additional 72 h before further evaluation.

Determination of cell protein content

Cell dishes were washed rapidly three times with Hanks solution. The cells were subsequently disrupted in 1% sodium dodecylsulfate (SDS), and the protein content was measured by the method of Lowry et al. (13).

Estimation of cell volume

Cardiomyocyte volume was calculated based on measurements of the cell diameters (14). The medium was aspirated, and cells were washed rapidly three times with D-Hanks solution containing 8 g/L NaCl, 0.4 g/L KCl, 0.06 g/L $Na_2HPO_4.H_2O$, 0.06 g/L KH_2PO_4, 0.35 g/L $NaHCO_3$, and 0.02 g/L phenol red. Cells were subsequently treated with 0.3 mL 0.1% Trypsin per well at 37°C for 10 min, and the digestion was subsequently stopped with 10% fetal calf serum (0.2 mL/well). Digested cells were collected and measured on an inverted microscope. For the measurements, four or five fields were randomly chosen and photographed at high power (400X), and 80 individual cell areas were evaluated using the CIAS Daheng image analysis system (China).

Incorporation of [^{3}H]-leucine

[^{3}H]-leucine uptake, which served as an index of protein synthesis, was determined as described by Luo et al. (15). The medium (DMEM, low glucose) was aspirated from myocardial cells grown on 24-well plates and replaced with 1 µCi [^{3}H]-leucine. Drugs were added and incubation was continued for 72 h. The medium was then aspirated, and cells were washed rapidly three times with cold Hanks solution. Next, the cells were lysed by the addition of 1 mL 1% SDS per well. Lysates were collected, precipitated by the addition of 1 mL 5% trichloroacetic acid and applied to fiberglass GF/C filters. After washing three times with 5 mL Hanks solution, the filters were dried and transferred to vials containing 4 mL scintillation fluid, and their radioactivity was determined by liquid scintillation counting. The radioactivity, which represented the [^{3}H]-leucine incorporated into newly synthesized protein, was expressed as cpm per well.

Measurement of cytosolic calcium transients

A spectrofluorimetric method was used to measure cytosolic Ca^{2+} transients using Fura-2/AM as the Ca^{2+} indicator. Cultured cardiomyocytes were incubated with Fura-2/AM (4 µM) in the medium for 25 min. To allow the de-esterification of Fura-2/AM in the cytosol, the loaded cells were maintained at 24-26°C for 60 min before $[Ca^{2+}]_i$ was measured. The cardiomyocytes were transferred to a superfusion chamber on the stage of an inverted microscope, which was coupled to a Till imaging system (Germany), and the cells were superfused with Hanks buffer. The emitted light was filtered at 510 nm. Fluorescence signals at 340 nm (F340) and 380 nm (F380) were recorded in a personal computer for data processing and analysis. At the end of each experiment, the Ca^{2+} ionophore ionomycin (20 µM) was added to each sample to estimate maximal fluorescence. Ethylene glycol tetracetic acid (EGTA) was added to a final concentration of 20 mM for the Ca^{2+}-free condition. Cytosolic $[Ca^{2+}]$ was calculated by the following formula: $[Ca^{2+}]_i = K_d \cdot (Sf_2 / Sb_2) \cdot (R_{340/380} - R_{min}) / (R_{max} - R_{340/380})$ (16), where K_d is the dissociation constant of Fura-2/AM for Ca^{2+} and was assumed to be 225 nM at 37°C. $R_{340/380}$ is the ratio of corrected fluorescence

signals. R_{max} is the ratio obtained after ionomycin treatment. R_{min} is the ratio of the corrected signals obtained after EGTA treatment. Sf_2 and Sb_2 represent the emission intensities at 380 nm excitation at saturation and under Ca^{2+}-free conditions, respectively.

Western blot analysis

Cells were diluted in buffer containing 65 mM Tris-HCl, pH 6.8, 3% SDS, 10% glycerol, and 6 M urea. After measurement of the protein concentration (BCA kit, Pierce, USA), β-mercaptoethanol and bromophenol blue were added to the buffer for electrophoresis. A volume containing 60 μg protein (for CaMKIIδ$_B$ or CaN) was separated on a 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (BioRad, USA). The blots were incubated at 4°C overnight with antibodies to CaMKIIδ$_B$ or CaN, and the resulting bands were detected using enhanced chemiluminescence. An antibody to CaMKIIδ$_B$ at Thr-276 (1:1000 dilution; Santa Cruz, USA) was used to detect the activated form of the kinase. The activated form of the phosphatase was evaluated with an antibody to CaN (1:2000 dilution; Santa Cruz). The intensities of the resulting bands were quantified using a CAMIAS008 image analysis system.

Statistical analysis

Data are reported as means ± SEM. Analysis of variance (one-way ANOVA) was used to compare the control and treatment groups. The Fisher least significant difference (LSD) test was used to determine differences between two groups. $P < 0.05$ was considered to be statistically significant.

Results

Effects of TNF-α on the protein content of cultured cardiomyocytes from neonatal rats

TNF-α induced a significant increase of protein content in a dose-dependent manner from 10-100 μg/L over a period of 72 h (Figure 1A). TNF-α (100 μg/L) also induced a significant increase of protein content in a time-dependent manner from 12 to 72 h (Figure 1B).

Effects of TNF-α on spontaneous $[Ca^{2+}]_i$ transients in neonatal rat cardiomyocytes

TNF-α (10-100 μg/L) significantly increased the amplitude (Figure 2A and B) of spontaneous $[Ca^{2+}]_i$ transients in cultured neonatal rat cardiomyocytes. None of the treatments had any effect on the resting $[Ca^{2+}]_i$ (Figure 2B) or the frequency (Figure 2C) of spontaneous $[Ca^{2+}]_i$ transients.

Effects of BAPTA on the increases in total protein content, [^{3}H]-leucine incorporation and cell size induced by TNF-α

TNF-α (100 μg/L) treatment significantly increased the total protein content (Figure 3A), [^{3}H]-leucine incorporation (Figure 3B) and cell size (Figure 3C) in cardiomyocytes. These effects were abolished by the addition of 4 μM BAPTA, which alone had no effect.

Effects of KN-93 and CsA on the increases in total protein content, [^{3}H]-leucine incorporation and cell size induced by TNF-α

TNF-α (100 μg/L) significantly increased the total protein content (Figure 4A), [^{3}H]-leucine incorporation (Figure 4B) and cell size (Figure 4C) in cardiomyocytes. These effects were abolished by the addition of 0.2 μM KN-93 or 0.2 μM CsA, neither of which had any effect in the absence of TNF-α.

Effects of TNF-α on CaMKIIδ$_B$ and CaN expression

TNF-α (100 μg/L) increased the expression of CaMKIIδ$_B$ and CaN in cardiomyocytes by 35.2 and 22.22%, respectively (Figures 5 and 6). These effects were abolished by the addition of 4 μM BAPTA, which itself had no effect.

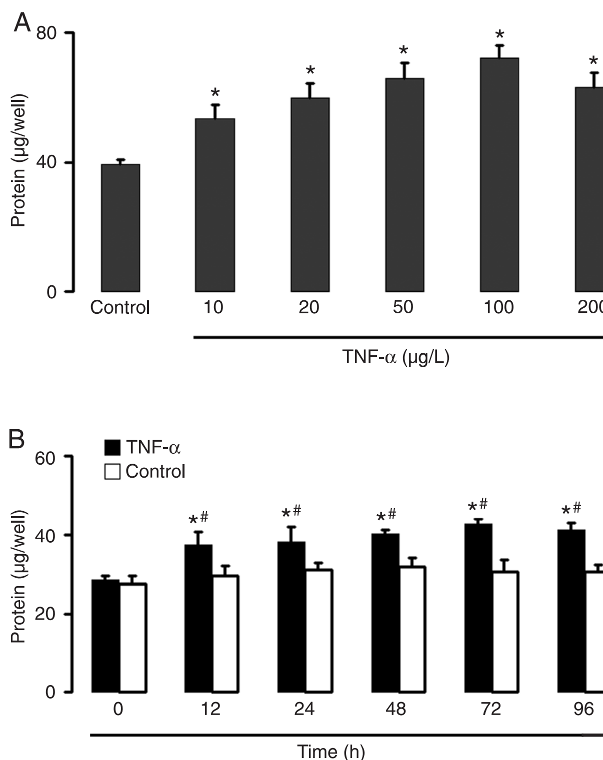


Figure 1. Effect of tumor necrosis factor alpha (TNF-α) concentration on total cellular protein content of cultured cardiomyocytes from neonatal rats at different doses of TNF-α for 72 h (*A*) and for different incubation times at 100 μg/L (*B*). Data are reported as means ± SEM for N = 8 per group. *P < 0.05 *vs* time-matched control. #P < 0.05 *vs* TNF-α (0 h; one-way ANOVA, post-LSD test).

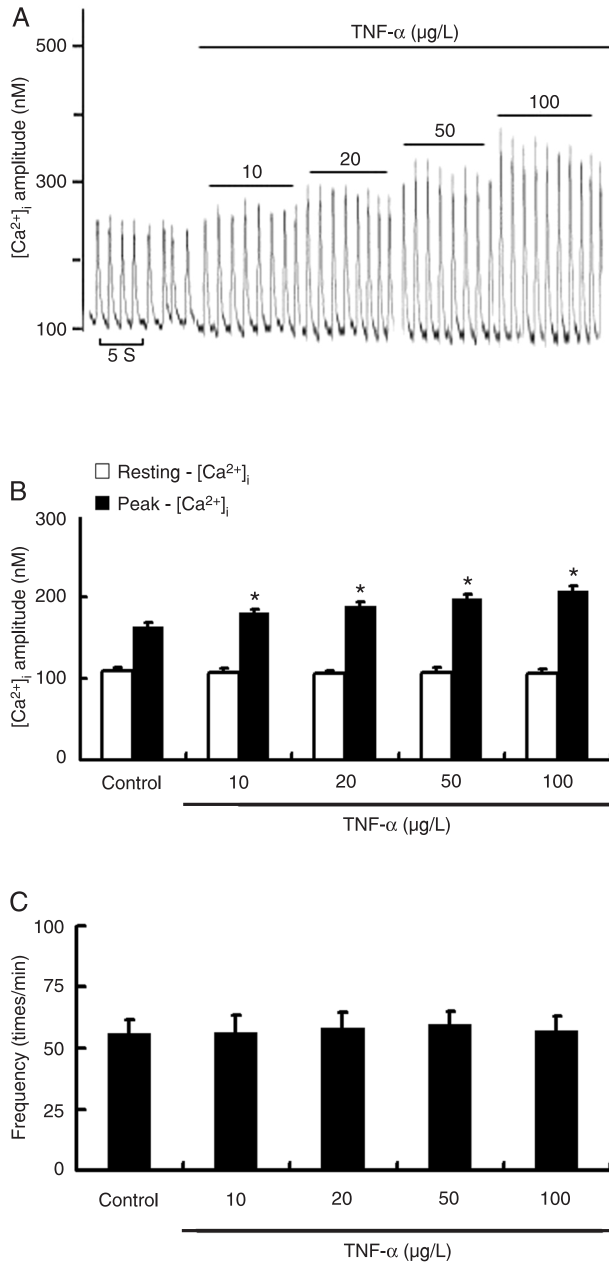


Figure 2. Effects of tumor necrosis factor alpha (TNF-α) on the peak amplitude, resting Ca^{2+} and frequency of the spontaneous $[Ca^{2+}]_i$ transients in cultured neonatal rat cardiomyocytes. *A*, Representative tracings. *B*, Peak amplitude and resting Ca^{2+} of the spontaneous $[Ca^{2+}]_i$ transients. *C,* Frequency of the spontaneous $[Ca^{2+}]_i$ transients. Cardiomyocytes were cultured in wells each covered with a coverslip. After the cells were cultured for 3 days, the coverslips with cardiomyocytes were incubated with Fura-2/AM at the concentration of 4 μM in the medium for 25 min. The unincorporated dye was removed by washing twice with fresh medium. The cytosolic calcium transients of multiple cells were then measured with the Till imaging system using a spectrofluorometric method. The different treatments were added at pre-established times. Data are reported as means ± SEM for N = 6 per group. *P < 0.05 *vs* control (one-way ANOVA, post-LSD test).

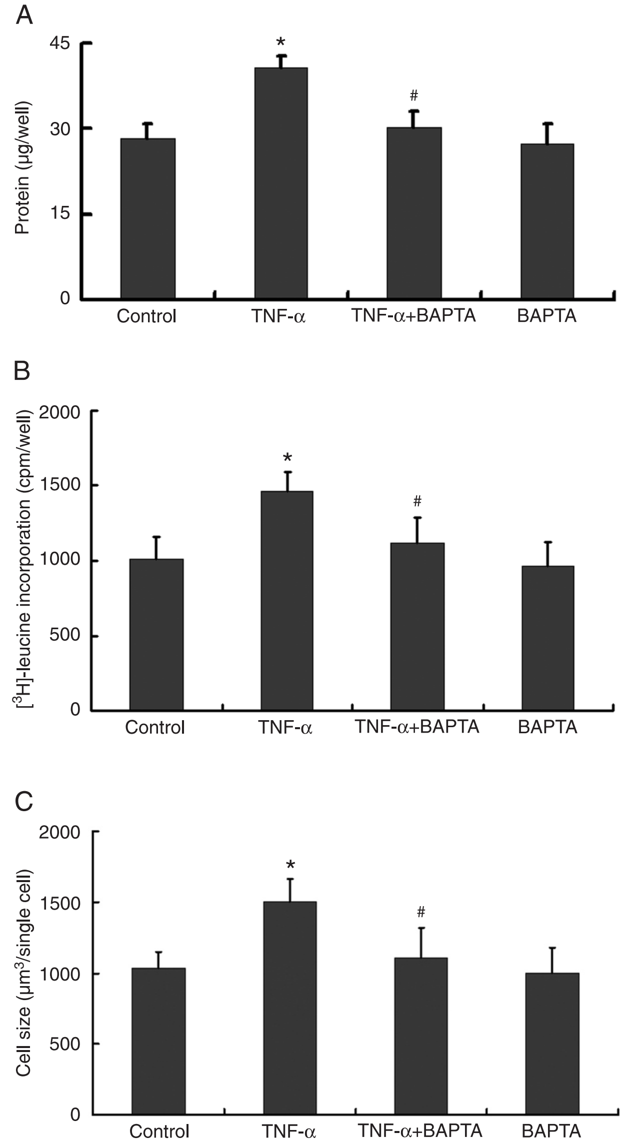
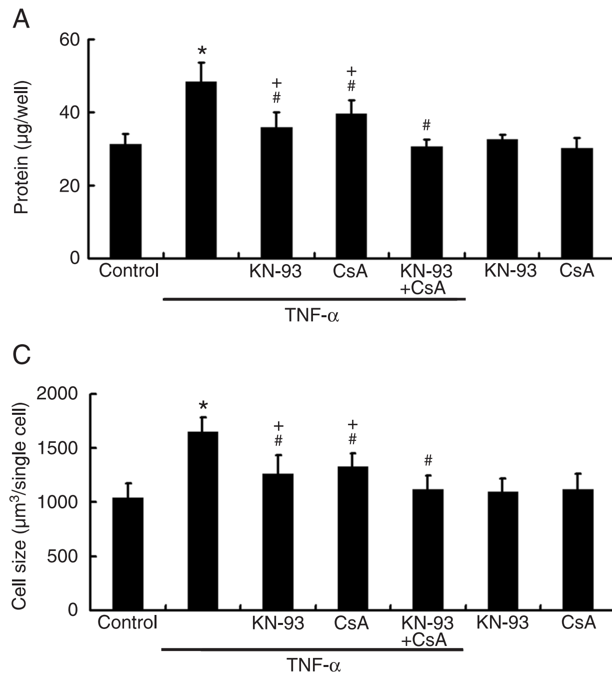


Figure 3. Effects of BAPTA (4 μM) on cellular protein content (A), $[^3H]$-leucine uptake (*B*) and cell size (*C*) of cultured neonatal rat cardiomyocytes treated with tumor necrosis factor alpha (100 μg/L TNF-α). Data are reported as means ± SEM; N = 8 for protein content and $[^3H]$-leucine uptake and N = 80 for cell size per group. *P < 0.05 *vs* control; #P < 0.05 *vs* TNF-α (one-way ANOVA, post-LSD test).

Discussion

TNF-α is a potent proinflammatory cytokine that is produced by several types of cells, including cardiomyocytes (17). The biological responses to TNF-α are mediated through two structurally distinct receptors: type 1 (TNFR1) and type 2 (TNFR2), both of which are expressed in cardiomyocytes (18). The results presented here indicate that



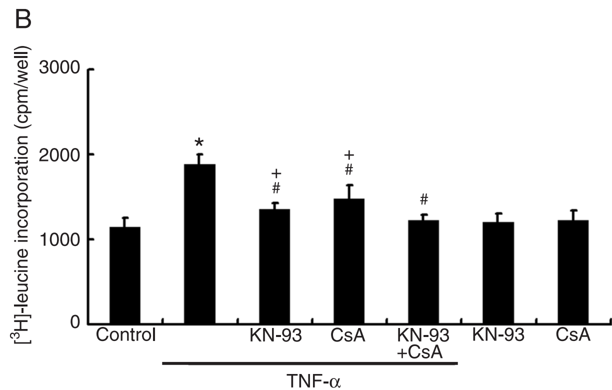


Figure 4. Effects of KN-93 (0.2 µM) and/or cyclosporine A (0.2 µM CsA) on the protein content (*A*), [^{3}H]-leucine uptake (*B*) and cell size (C) of cultured neonatal rat cardiomyocytes treated with tumor necrosis factor alpha (100 µg/L TNF-α). Data are reported as means ± SEM; N = 8 for protein content and [^{3}H]-leucine uptake and N = 80 for cell size per group. *$P < 0.05$ *vs* control; $^{\#}P < 0.05$ *vs* TNF-α. $^{+}P < 0.05$ *vs* TNF-α + KN93 + CsA (one-way ANOVA, post-LSD test).

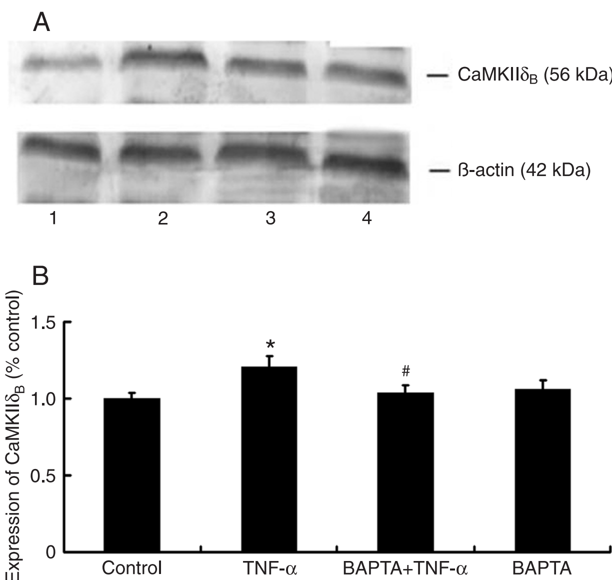


Figure 5. Effects of tumor necrosis factor alpha (TNF-α) on Ca^{2+}/calmodulin-dependent kinase II (CaMKIIδ_B) expression in cultured neonatal rat cardiomyocytes. *A*, Representative autoradiograms of CaMKIIδ_B and β-actin. *Lane 1* = Control; *lane* 2 = TNF-α (100 µg/L); *lane 3* = TNF-α (100 µg/L) + BAPTA (4 µM); *lane 4* = BAPTA (4 µM). *B,* Relative levels of CaMKIIδ_B expressed as the absorbance ratio of each group (% control). The expression of CaMKIIδ_B increased significantly only in TNF-α (100 µg/L) compared to control. Data are reported as means ± SEM for N = 4 per group. *$P < 0.05$ *vs* control. $^{\#}P < 0.05$ *vs* TNF-α (one-way ANOVA, post-LSD test).

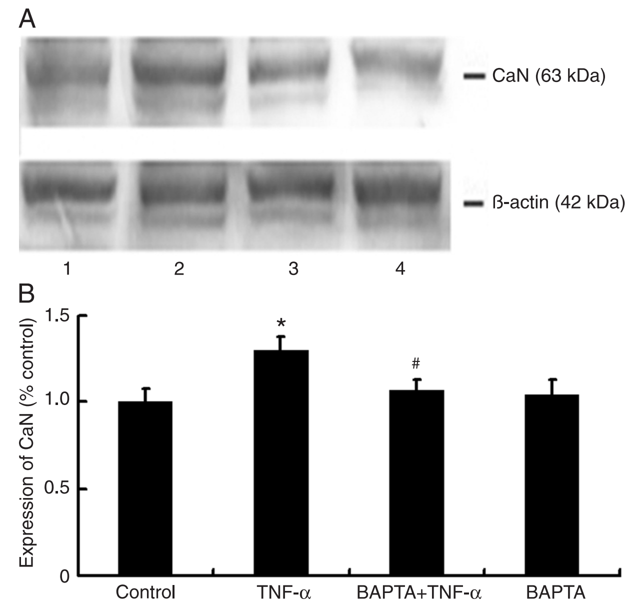


Figure 6. Effects of tumor necrosis factor alpha (TNF-α) on calcineurin (CaN) expression in cultured neonatal rat cardiomyocytes. *A*, Representative autoradiograms of CaN and β-actin. *Lane 1* = Control; *lane 2* = TNF-α (100 µg/L); *lane* 3 = TNF-α (100 µg/L) + BAPTA (4 µM); *lane* 4 = BAPTA (4 µM). *B*, Relative levels of CaN expressed as the absorbance ratio of each group (% control). The expression of CaN increased significantly only in TNF-α (100 µg/L) compared to control. Data are reported as means ± SEM for N = 4 per group. *$P < 0.05$ *vs* control, $^{\#}P < 0.05$ *vs* TNF-α (one-way ANOVA, post-LSD test).

the heart is not only a site of TNF-α synthesis but is also a target of TNF-α activity.

Recent studies have shown that circulating levels of TNF-α are elevated in patients with chronic heart failure, such as ischemic heart disease and dilated cardiomyopathy (3). Myocardial hypertrophy is one of the principal features of such cardiac diseases (19).

In the present study, we observed an increase in protein content, cell size and [^{3}H]-leucine uptake in TNF-α-stimulated cardiomyocytes, which confirms that TNF-α induces myocardial hypertrophy. Our results are consistent with those of previous studies (3). TNF-α treatment led to a significant increase in protein content in a dose-dependent manner from 10 to 100 µg/L. TNF-α also induced a significant increase of protein content in a time-dependent manner from 12 to 72 h. The TNF-α concentrations used in the subsequent experiments in the present study were based on these initial results.

Krown et al. (20) reported for the first time that TNF-α inhibited the L-type Ca^{2+} current ($I_{Ca,L}$) and Ca^{2+} transients. Li et al. (9) discovered that TNF-α inhibited $I_{Ca,L}$ in rat ventricular myocytes and increased the intercellular free Ca^{2+} concentration by stimulating the release of Ca^{2+} from intracellular stores. In the last decade, Defer et al. (18) and Amadou et al. (21) demonstrated that TNF-α exhibited both positive and negative activities on [Ca^{2+}] transients.

We showed that 100 µg/L TNF-α significantly increased the amplitude of spontaneous Ca^{2+} transients in cultured cardiomyocytes isolated from neonatal rats. None of the TNF-α treatments had any effect on the resting $[Ca^{2+}]_i$ and frequency of the spontaneous Ca^{2+} transients. These results suggest that TNF-α induced an increase of the amplitude of the spontaneous Ca^{2+} transients, but did not change the frequency of Ca^{2+} transients.

Previous studies have suggested that intracellular Ca^{2+} may be involved in the myocardial hypertrophy induced by TNF-α. Consistent with this hypothesis, we found that the effects of TNF-α (100 µg/L) on total protein content, cell size and [^{3}H]-leucine incorporation were abolished in the presence of BAPTA, an intracellular Ca^{2+} chelator. These results indicated that calcium plays a critical role in the myocardial hypertrophy induced by TNF-α, suggesting that the TNF-α-induced hypertrophic response may be linked to Ca^{2+} homeostasis.

One key issue is the mechanism of Ca^{2+} involvement in TNF-α-induced hypertrophy in cardiac myocytes. It is well known that both major calcium effectors, Ca^{2+}/CaMKII and CaN, play critical roles in the development of myocardial hypertrophy induced by several hypertrophic agents such as endothelin-1 and angiotensin II, or pressure overload. Further studies are expected to determine whether these pathways are also involved in TNF-α-induced cardiac hypertrophy. Our pharmacological studies using specific inhibitors revealed that pretreatment with either KN-93 or CsA abolished the TNF-α-induced increase in protein content, protein synthesis, and cell size in cultured neonatal rat cardiomyocytes, suggesting that both CaMKII and CaN are involved in the myocardial hypertrophy induced by TNF-α. In addition, increased CaMKII$δ_B$ and CaN expression was also induced by TNF-α, and this increase was abolished by the addition of 4 µM BAPTA. All of these data strongly suggest that Ca^{2+} plays a critical role in the myocardial hypertrophy induced by TNF-α through CaMKII and CaN and that the isoform of CaMKII that is involved may be CaMKII$δ_B$. We conclude that myocardial hypertrophy is induced by TNF-α through a two-step pathway: TNF-α first increases intracellular Ca^{2+}, which subsequently induces an increase in the expression of CaMKII$δ_B$ and CaN.

The study provides evidence confirming that Ca^{2+}/CaMKII and CaN are involved in myocardial hypertrophy induced by TNF-α.

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